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Unique Gene Expression Profiles in Infants Vaccinated with Different Strains of *Mycobacterium bovis* Bacille Calmette-Guérin[▽]

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Vaccination with *Mycobacterium bovis* bacille Calmette-Guérin (BCG) has variable efficacy in preventing tuberculosis. We hypothesized that some of this variation might be due to differences among BCG strains. To test this, neonates in Orizaba, Mexico, were vaccinated with one of three different BCG strains (BCG-Brazil [BBCG], BCG-Denmark [DBCG], or BCG-Japan [JBCG]). One year after vaccination, peripheral blood mononuclear cells (PBMC) were obtained and recall immune responses to culture filtrate proteins (CFP) of *Mycobacterium tuberculosis* were evaluated using quantitative real-time PCR. CFP-activated PBMC from BBCG- and DBCG-immunized children expressed high levels of cytokines characteristic of an adaptive immune response (gamma interferon, interleukin-2β [IL-12β], and IL-27), while those from children immunized with JBCG did not. In contrast, vaccination with JBCG resulted in significantly greater expression of cytokines characteristic of a proinflammatory immune response (IL-1α, IL-1β, IL-6, and IL-24) in PBMC activated with CFP compared to PBMC from children vaccinated with BBCG or DBCG. Thus, different strains of BCG can activate different immune pathways, which may affect long-term vaccine efficacy.

It is estimated that approximately one-third of the world's population is infected with *Mycobacterium tuberculosis*, with 8 million new cases and nearly 3 million deaths occurring annually (30, 38). *Mycobacterium bovis* bacille Calmete-Guérin (BCG), the most widely used vaccine in the world, is highly effective at reducing the risk of disseminated forms of tuberculosis in early childhood (11). However, studies have shown that its protection against adult tuberculosis is at best variable and often negligible (17). Variable BCG efficacy has been attributed to geographical differences (17), a masking effect due to an environmental mycobacterium (14), host genetic factors, or variations in BCG vaccine strains.

Genomic analysis of BCG vaccines demonstrated that there are numerous genetic differences among the strains, including single-nucleotide polymorphisms, duplications, and deletions. The impact of these differences on the protective efficacy of BCG has not been determined. Functional studies comparing BCG immunogenicity and efficacy in laboratory animal models are subject to considerable controversy. In a mouse model, different BCG strains induced various levels of protection, with Japanese BCG (JBCG) unable to provide protection against mycobacterial challenge (7, 32). However, in a guinea pig model, both JBCG and Danish BCG (DBCG) provided good protection (43). In a recent study in Africa, vaccination with JBCG resulted in greater secretion of Th1 cytokines gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin-2 (IL-2) than vaccination with DBCG (12).

Central to the development of tuberculosis is the inability of *M. tuberculosis*-infected macrophages to contain the pathogen and the failure of T cells to confer long-lasting protective immunity. Therefore, the efficacy of BCG is associated with not only innate but also adaptive immune responses. In this study, we evaluated *M. tuberculosis*-specific recall immune response in infants vaccinated 1 year earlier with one of three BCG strains. After stimulation of peripheral blood mononuclear cells (PBMC) with culture filtrate proteins (CFP), mRNA for a panel of immune-related genes was monitored by quantitative real-time PCR. PBMC from children immunized with Brazilian BCG (BBCG) and DBCG expressed higher levels of IL-12β, IL-27, and IFN-γ in response to CFP, while those from children immunized with JBCG expressed higher levels of IL-1α, IL-1β, IL-6, and IL-24.

MATERIALS AND METHODS

Participants. Healthy neonates were recruited at a community-based, general hospital in Veracruz, Mexico. Exclusion criteria for enrollment were birth weight below 2,500 g, a family history of tuberculosis, and known human immunodeficiency virus infection. Infants were immunized intradermally with one of three BCG strains. BBCG (BCG Moreau; Fundacao Ataulpho de Paiva, Brazil) is used in the vaccination program for neonates in Brazil. DBCG (BCG Danish 1331; Statens Serum Institute) and JBCG (BCG Tokyo 172; Japan BCG Laboratory) are used in the vaccination program in Mexico. Neonates were vaccinated with JBCG from 26 April 2003 to 29 September 2003, with the BBCG from 11 November 2003 to 9 December 2003, and with the DBCG from 10 December 2003 to 7 March 2004. The dose of each vaccine was 0.1 ml, as recommended by the manufacturer and used by the Mexican and Brazilian vaccination programs. A total of 107 neonates were recruited, and 36 were vaccinated with BBCG, 35 were vaccinated with DBCG, and 36 were vaccinated with JBCG. At 1 year, peripheral blood was obtained, and PBMC were purified by density gradient

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TABLE 1. Characteristics of BCG-immunized newborns[a]

Vaccine (*n*)	No. (%) of males	Mean age at vaccination (wk) ± SD	Mean wt (kg) ± SD	Mean length (cm) ± SD	Mean age (yr) when blood drawn
BBCG (36)	22 (61.1)	0.37 ± 0.87	3.04 ± 0.37	50.81 ± 2.12	1.03 ± 0.07
DBCG (35)	18 (51.4)	0.10 ± 0.08	3.05 ± 0.39	50.85 ± 2.46	1.03 ± 0.05
JBCG (36)	21 (58.3)	0.10 ± 0.10	3.05 ± 0.42	49.94 ± 2.61	1.08 ± 0.16

[a] For each characteristic, the chi-square test or one-way analysis of variance yielded a P value of >0.05.

centrifugation using lymphocyte separation medium (MP Biomedicals, Aurora, OH). PBMC were cryopreserved in 90% fetal bovine serum–10% dimethyl sulfoxide, stored in liquid nitrogen, and shipped to Stanford University on dry ice. Informed consent was obtained from parents or guardians at the time of immunization and when blood was drawn. The study was approved by the institutional review boards of all participating institutions.

PBMC activation and real-time PCR assay. PBMC were cultured in RPMI 1640 medium (GIBCO, NY) supplemented with L-glutamine, penicillin-streptomycin, nonessential amino acids (GIBCO, NY), sodium pyruvate (Irvine Scientific, CA), and 10% heat-inactivated pooled human serum (Gemini, Woodland, CA) in the absence or presence of 10 μg/ml CFP (Research Materials and Vaccine Testing Center, Colorado State University) for 15 h. Total RNA was isolated from PBMC using RNeasy minikits (QIAGEN, Santa Clarita, CA) and was transcribed into cDNA (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Expression of 17 immune-related genes was analyzed using quantitative PCR with an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA) (details of PCR protocol at www.appliedbiosystems.com). All primers were purchased from Applied Biosystems, including the FOXP3, IFN-γ, IL-1α, IL-1β, IL-2, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12α, IL-12β, IL-23α, IL-24, IL-27, TNF-α, and transforming growth factor β1 (TGF-β1) gene primers. The expression level of a gene in a given sample was represented as the fold increase $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_{T(\text{sample, stimulated})} - \Delta C_{T(\text{sample, unstimulated})}$ and $\Delta C_T = C_{T(\text{sample})} - C_{T(\text{GUS})}$, where GUS is the housekeeping gene encoding β-glucoronidase and C_T is the threshold cycle.

Statistical analysis. Results are expressed as increases in CFP-stimulated mRNA levels. Levels of mRNA expression among newborns were compared using the Kruskal-Wallis test. Dunn's posttest was used to determine whether responses from BBCG- or DBCG-immunized neonates differ from those immunized with JBCG. To explore mRNA expression patterns, cluster analysis was conducted to identify the similarities and differences among individuals. Cytokines with statistically different mRNA expression levels between neonates immunized with BBCG or DBCG and those immunized with JBCG were selected for hierarchical clustering analysis. Ward's minimum-variance clustering was performed on both individuals and gene mRNA expression levels. The cluster is color coded, using red for high expression and blue for low expression, and is shown as a dendrogram.

RESULTS

The characteristics of the 107 infants included in this study are summarized in Table 1. There are no significant differences among the three groups. At follow-up, two cases of tuberculosis had occurred among family members in the household (one in the DBCG-immunized group and one in the JBCG-immunized group). The two infants who came into contact with individuals with tuberculosis were excluded from this analysis. None of the children developed active tuberculosis.

PBMC from BBCG- and DBCG-vaccinated infants express higher levels of IL-12β, IL-27, and IFN-γ mRNA, while those from JBCG-vaccinated newborns express higher levels of IL-1α, IL-1β, IL-6, and IL-24 mRNA, in response to CFP. No significant differences in the increases in any of the genes tested were observed in PBMC from neonates immunized with DBCG or BBCG (Fig. 1 to 3). In contrast, expression of 7 of 17 genes differs significantly between PBMC of neonates immunized with JBCG and those of neonates immunized with BBCG or DBCG (Fig. 1 and 2). IFN-γ is a critical cytokine associated with protection against tuberculosis. Humans who have defects in the genes encoding IFN-γ or the IFN-γ receptor are susceptible to serious mycobacterial infections. Increases in IFN-γ mRNA are higher in CFP-stimulated PBMC from infants vaccinated with BBCG ($P < 0.01$) or DBCG ($P < 0.05$) than in those from infants vaccinated with JBCG (Fig. 1A).

We also analyzed expression of IFN-γ-inducing cytokines, including IL-12α, IL-12β, IL-23α, and IL-27, which are mainly produced by monocytes and dendritic cells. IL-12 is a heterodimer composed of two subunits, p35 and p40, encoded by the IL-12α and IL-12β genes, respectively (13). Levels of IL-12β and IL-27 (Fig. 1B and C) but not IL-12α and IL-23α (Fig. 3G and H) were significantly increased in PBMC from infants vaccinated with BBCG ($P < 0.001$) or DBCG ($P < 0.001$) compared to those from infants immunized with JBCG. Expression of IL-1α, IL-1β, IL-6, and IL-24 was increased in

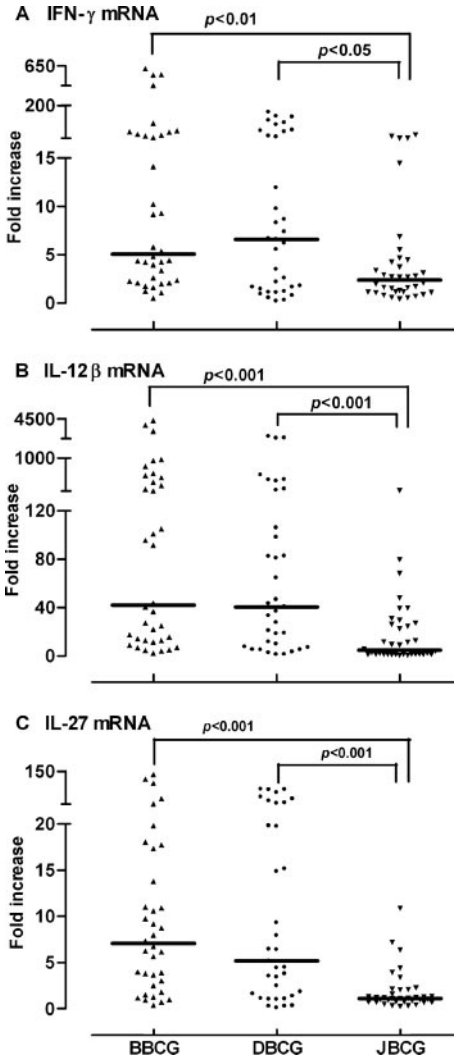


FIG. 1. Expression of IFN-γ, IL-12β, and IL-27 mRNA in PBMC from infants vaccinated with BBCG, DBCG, or JBCG. Median increases are represented by horizontal bars.

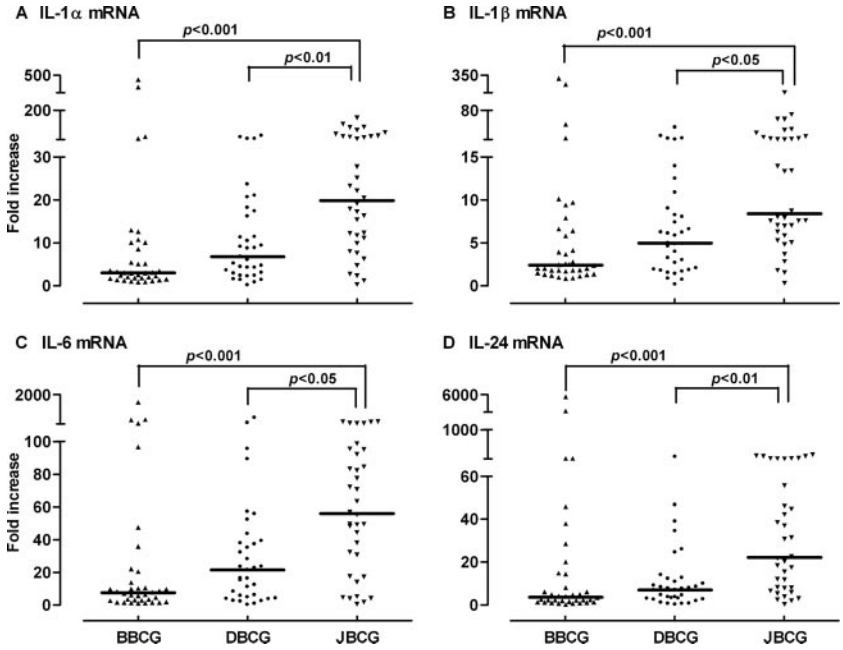


FIG. 2. Expression of IL-1α, IL-1β, IL-6, and IL-24 in PBMC from infants vaccinated with BBCG, DBCG, or JBCG. Median increases are represented by horizontal bars.

PBMC from JBCG-vaccinated infants compared to those immunized with BBCG or DBCG (Fig. 2A to D).

FOXP3, IL-10, TGF-β1, IL-2, IL-5, IL-8, IL-9, and TNF-α are expressed similarly in the three groups of BCG-vaccinated newborns. Recently, regulatory T cells, including $FOXP3^+$ $CD4^+$ $CD25^+$ cells (23), IL-10-producing Tr1 cells (5), and TGF-β-producing Th3 cells (25), have been implicated in *M. tuberculosis* infection and development of tuberculosis. Therefore, we measured expression of FOXP3, IL-10, and TGF-β1 to examine whether the variable efficacy of BCG might be due to induction of regulatory T cells. There was no increase in TGF-β1 (Fig. 3I) and only a minor increase in FOXP3 (Fig. 3A). Levels of IL-10 mRNA were similarly elevated in all three groups (Fig. 3F). In addition, no differences in levels of expression of IL-2, IL-5, IL-8, IL-9, and TNF-α were observed (Fig. 3B to E and J).

Two group of cytokines were identified by the cluster method. The seven genes with significant differential expression in PBMC from BBCG- or DBCG-immunized versus JBCG-immunized neonates were subjected to cluster analysis. Hierarchical clustering analysis was performed for each individual and for each cytokine. As shown in Fig. 4A, the seven cytokine genes can be divided into two major subclusters, characterized by increased expression of IFN-γ/IL-27/IL-12β or increased expression of IL-1α/IL-1β/IL-24/IL-6, indicating that the two types of immune responses can be separated based on their global gene expression profiles.

Although diverse patterns of individual mRNA expression were observed, individuals could be segregated into low responders (neonates with increased mRNA levels for no genes or one gene) and high responders (neonates with increased mRNA levels for two to seven genes). There was no correlation between the strain of BCG used for immunization and high or low responders (Fig. 4B; $P > 0.05$). The cytokine responses in high responders were further divided into three subtypes: type I includes those individuals expressing IL-1α, IL-1β, IL-24, and IL-6 genes; type II includes those individuals expressing IFN-γ, IL-27, and IL-12β genes; and type III includes those individuals expressing all seven genes. Further analysis revealed that 16/18 (89%) of the neonates in type I were vaccinated with JBCG and 19/19 (100%) of the neonates in type II were vaccinated with BBCG or DBCG. In type III, all four newborns were vaccinated with DBCG (Fig. 4C). A significant difference between type I and type II/III cytokine responses ($P < 0.0001$) was observed.

DISCUSSION

In this study, we examined *M. tuberculosis*-specific recall immune responses in newborns vaccinated with one of three different strains of BCG. CFP, a mixture of secreted proteins from cultured *M. tuberculosis*, contains highly immunogenic antigens that have been associated with protective immunity against tuberculosis (35, 37). We identified two classes of BCG based on the distinct gene expression profiles induced in response to CFP. Our results suggest that vaccination with BBCG or DBCG preferentially induces cytokines involved in adaptive immunity (IL-12/IL-27/IFN-γ) while vaccination with JBCG preferentially induces cytokines associated with proinflammatory responses (IL-1α/IL-1β/IL-6/IL-24). These results provide new insights into the variable efficacy of BCG in protection against tuberculosis.

The central role of Th1 cytokines in protection against tuberculosis has been well established. Production of IL-12 by dendritic cells is associated with IFN-γ secretion by Th1 $CD4^+$ T cells after antigenic stimulation and is important in mycobacterial immunity (33). Dendritic cells, but not macrophages, can deliver *M. tuberculosis* antigens from the lung to draining

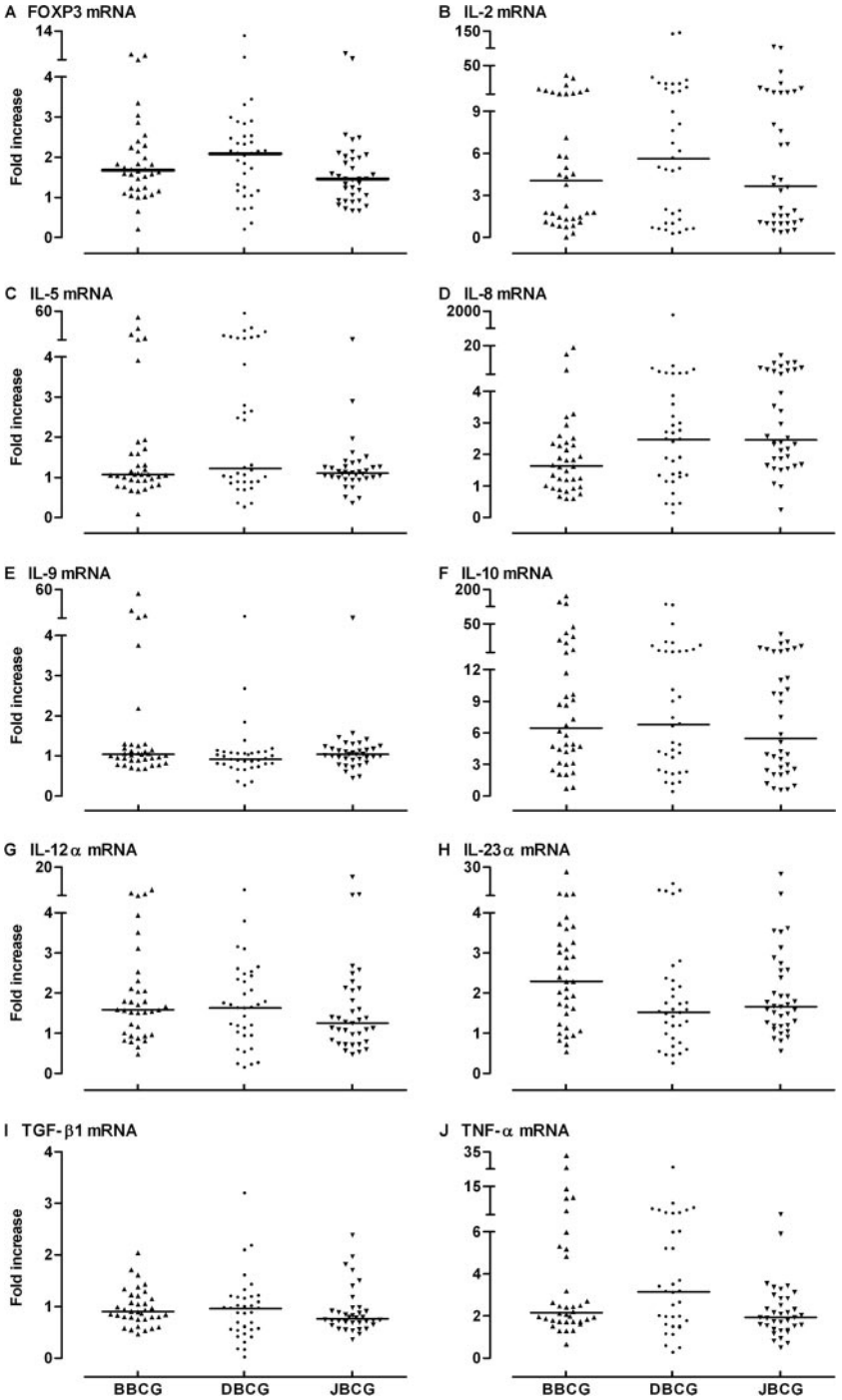
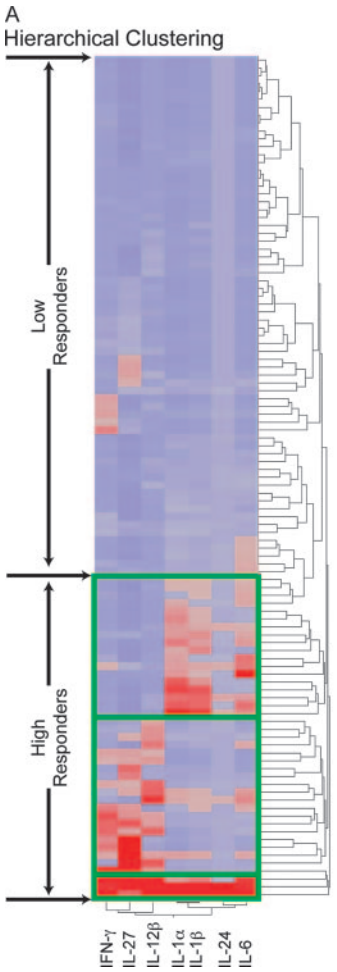


FIG. 3. Expression of FOXP3, IL-2, IL-5, IL-8, IL-9, IL-10, IL-12α, IL-23α, TGF-β1, and TNF-α in PBMC from infants vaccinated with BBCG, DBCG, or JBCG. Median increases are represented by horizontal bars.

lymph nodes to initiate a Th1 immune response, which is most likely mediated by CCR7 (3) and CCR5 (1). In addition, dendritic cells can directly produce IFN-γ in response to BCG stimulation (20). IL-27, a member of IL-12 family, increases the production of IFN-γ by naive $CD4^+$ T cells (9). The role of IL-27 in *M. tuberculosis* infection is still unclear. In mice infected with *M. tuberculosis*, IL-27 both prevents optimal antimycobacterial protection and limits the pathological sequelae of chronic inflammation (27).

Production of proinflammatory cytokines, including TNF-α, IL-1, and IL-6, is an alternative pathway in defense against *M. tuberculosis* infection (22). TNF-α has multiple immune and pathological roles in tuberculosis (18). One of its important functions is in granuloma formation (2). We did not observe



B
Low vs. high responders

Responders	BBCG	DBCG	JBCG	Total
Low	25	21	20	66
High	11	14	16	41
Total	36	35	36	107

C
Cytokine responses in high responders

Type	BBCG/DBCG	JBCG	Total
I	2	16	18
II	19	0	19
III	4	0	4
Total	25	16	41

FIG. 4. Hierarchical clustering of expression of IFN-γ, IL-27, IL-12β, IL-1α, IL-1β, IL-24, and IL-6. The color intensity reflects the magnitude of high (red rectangle) or low (blue rectangle) mRNA expression. Each row represents the mRNA expression profile of one infant, and each column represents one cytokine. (A) Hierarchical clustering of mRNA expression. Cytokines with similar immune functions are grouped. (B) Distribution of high and low responders among BCG-vaccinated infants. The chi-square test was performed ($P > 0.05$). (C) Cytokine responses in high responders among BBCG- or DBCG-vaccinated and JBCG-vaccinated infants. Type I, IL-1α/IL-1β/IL-24/IL-6; type II, IFN-γ/IL-27/IL-12β; type III, IFN-γ/IL-27/IL-12β/IL-1α/IL-1β/IL-24/IL-6. Fisher's exact test was performed ($P < 0.0001$).

any difference in TNF-α expression among the three groups vaccinated with different strains of BCG. IL-1, in particular IL-1β, is associated with protective immunity against mycobacteria (28). Mice with defective IL-1 receptors express low levels of nitric oxide (44) and reduced IFN-γ (29). IL-6, which has both pro- and anti-inflammatory properties, is produced relatively early at the site of *M. tuberculosis* infection (26, 34). Although IL-6 inhibits the production of TNF-α and IL-1β, thereby enhancing *M. tuberculosis* growth (39), IL-6-deficient mice display increased susceptibility to infection with *M. tuberculosis* (31). We found that both IL-6 and IL-1 (α and β) were significantly elevated in CFP-stimulated PBMC from JBCG-vaccinated infants, suggesting a protective role for IL-6. Recently it was reported that IL-6 also directly affects regulatory T cells (15, 36). $CD4^+$ $CD25^+$ regulatory T cells but not $CD4^+$ $CD25^-$ T cells selectively express the IL-6R alpha chain and show IL-6-dependent STAT-3 phosphorylation (16). Thus, the high levels of IL-6 in JBCG-vaccinated infants may serve to suppress their antigen-specific regulatory T cells.

We show here that immunization with JBCG preferentially induces memory cells producing cytokines involved in proinflammatory responses. Interestingly, increased IL-24 mRNA expression was observed in the group vaccinated with JBCG. IL-24 is a member of the IL-10 family of cytokines and is mainly produced by activated monocytes and Th2 cells (41). IL-24 plays a role in cell proliferation (40), tumor cell apoptosis (42), and antiviral immunity (21). IL-24 induces the expression of TNF-α and IL-6 by PBMC (8). The function of IL-24 in tuberculosis is unknown.

Increases in regulatory T cells, including Tr1, Th3, and $CD4^+$ $CD25^+$ $FOXP3^+$ cells, have been reported in adult tuberculosis patients (5, 23, 25). Recently, a moderate upregulation of FOXP3 mRNA was also observed in BCG-vaccinated newborns (24). In our study, we observed a significant increase in IL-10, but not in FOXP3 or TGF-β1, mRNA levels in response to CFP. However, no differences were observed among newborns vaccinated with different strains of BCG.

Our result differ from those of Davids et al., who reported

that vaccination with JBCG induced higher numbers of IFN-γ-secreting cells than did vaccination with DBCG (12). In that study, infants were tested 10 weeks after vaccination, whereas in our study, there was a 12-month interval between vaccination and testing. In addition, Davids and colleagues used BCG to restimulate cells, while we used CFP. Most previous studies assessing BCG efficacy have monitored induction of IFN-γ (4). Although it is clear that defective IFN-γ responses render the host susceptible to *M. tuberculosis* infection (6), IFN-γ alone is insufficient to protect against development of tuberculosis (19). Moreover, high expression of IFN-γ induced by BCG may not be associated with improved prevention of tuberculosis. In a review of the literature, Colditz et al. found that BCG vaccination of infants reduced the risk of tuberculosis by over 50% regardless of the BCG strain, and this reduction was observed for many different populations, study designs, and forms of tuberculosis (10).

Our data suggest that different strains of BCG can induce distinct but broad ranges of immunologic responses. Although our findings do not address the protective efficacy of each BCG strain, it is clear that long-term follow-up of individuals vaccinated with different strains of BCG is needed. Only that type of study will allow comparison of the immunity induced by each BCG strain and identification of immune components associated with protection against tuberculosis.

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